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Ovine colostrum nanopeptide affects amyloid beta aggregation

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ABSTRACT

A colostral proline-rich polypeptide complex (PRP) consisting of over 30 peptides shows beneficial effects in Alzheimer's disease (AD) patients when administered in the form of sublinqual tablets called Colostrinin. The aim of the present studies was to investigate whether nanopeptide fragment of PRP (NP) – one of the PRP complex components can affect aggregation of amyloid β (Aβ1-42). The effect of NP on Aβ aggregation was studied using Thioflavin T (ThT) binding, atomic force microscopy, and analyzing circular dichroism spectra. Results presented suggest that NP can directly interact with amyloid beta, inhibit its aggregation and disrupt existing aggregates acting as a β sheet breaker and reduce toxicity induced by aggregated forms of Aβ.

1. Introduction

A proline-rich polypeptide complex (PRP) first isolated from ovine colostrum and subsequently found in human and cow colostra is composed of a mixture of peptides of various molecular masses ranging from 500–3000 kDa. PRP contains a high proportion of proline residues and hydrophobic amino-acids 25% and 40%, respectively [1–4].

The PRP complex has shown immunoregulatory properties, inducing the maturation and differentiation of murine thymocytes, and affecting humoral and cellular immune responses, both in vitro and in vivo. The polypeptide is not species-specific and is active in mice, humans, chickens, and rats [2,5–9]. A nanopeptide (NP) fragment of the PRP complex: Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro, both isolated from the chymotrypytic digestion products and obtained by chemical synthesis shows in mice the full spectrum of immunotropic activities of the untreated ovine PRP [10,11].

The PRP complex affects the differentiation/maturation process of cells of monocyte/macrophage lineage [12] and shows regulatory effects on secretion of NO [13], reactive oxygen species, and cytokines [14,15]. PRP affects both, innate and adaptive immune responses and seems to restore balance in cellular immune functions.

PRP and NP show beneficial effects on the cognitive functions in older rats [9,16] and PRP improve mood and cognitive abilities in humans [8]. PRP, in the form of sublinqual tablets called Colostrinin, containing 100 μg of PRP, improves the outcome of Alzheimer's disease (AD) patients and prevents further deterioration of the patients' health status [17–19].

Deposits of amyloid β protein in neurite plaques and cerebral vessels are a pathological hallmark of AD. Aggregates and fibrilar amyloid β (Aβ) deposits are closely associated with inflammatory responses, activation of microglia. Aβ stimulates the glial overproduction of interleukins, ROS, and NO [20–23]. One of attractive therapeutic approaches to AD is use of preparations which may inhibit aggregation and formation of insoluble deposits of Aβ in the brain or preparations which may disrupt aggregates of Aβ already formed [24–26] and prevent its toxicity [27,28].

The mechanism of action of PRP in AD patients is not yet fully clarified. It was of interest to find out whether PRP and its component peptides, besides regulatory properties in inflammatory processes, can affect formation of aggregates of Aβ and thus reduce the cytotoxic effects of the amyloid.

As the PRP complex is a mixture of over 30 peptides, in our studies we used the nanopeptide NP, one of its components showing similarity to PRP in regulation of immune and cognitive

Abbreviations: PRP, proline-rich polypeptide complex from ovine colostrum; NP, nanopeptide fragment of PRP; AD, Alzheimer's disease; Aβ, amyloid β; ThT, Thioflavin T; CD, circular dichroism; AFM, atomic force microscopy

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processes. The effect of the peptide on Aβ aggregation was studied using Thioflavin T (ThT) binding, atomic force microscopy, and analysing circular dichroism spectra (CD). The results obtained showed that NP acts as a β-sheet breaker and may inhibit Aβ aggregation and disrupt existing aggregates.

2. Materials and methods

2.1. Aggregation of amyloid beta 1-42 (Aβ1-42) and effect of NP

Aβ1-42 was purchased from Bachem, Switzerland. NP: Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro was synthesized by a solid-phase method and kindly provided by Professor G. Kupryszewski from the Institute of Chemistry, University of Gdańsk.

One milligrams samples of commercial preparations of Aβ1-42 were dissolved in 1 ml of 1,1,1,3,3,3-hexafluoro-2propanol (HFIP) (Sigma, USA). If necessary, the samples were sonicated in ice-water bath for 10 min. Aliquots containing 100 μg of Aβ were dried in a SpeedVac and stored at −80 °C. For experiments, the dried samples were dissolved in 300 μl of water (MiliQ) or PBS (final concentration of Aβ 74 μM) and incubated for various times at 37 °C to induce aggregation of Aβ and formation of fibrillar oligomeric species. To measure the effect of NP, 100 μg samples of Aβ were dissolved in 150 μl of water or PBS, mixed with 150 μl of solution of NP and incubated at 37 °C. The final molar concentration of Aβ was 74 μM and the proportion of NP to Aβ was 5:1 or 1:1. At various times, aliquots of Aβ solutions without and with NP were withdrawn for examination using atomic force microscopy (AFM), CD, and ThT binding. The concentrations of the nonaggregated, oligomeric, and fibrilar Aβ are based on the initial peptide mass.

2.2. Cell viability

Rat pheochromocytoma (PC12) cells were cultured in 75-cm^2 flasks at 37 °C in 95% humidified atmosphere and 5% CO_2 in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine (Gibco, England) and 5% horse serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine (Sigma, USA). Viability of cells incubated 72 h with Aβ samples aggregated in the presence and in the absence of NP was assessed by the trypan blue exclusion assay. Cells (1×10^4) were treated with 0.4% trypan blue stain for 15 min. Trypan blue excluding cells (live) and stained (dead) cells were counted in a haemocytometer in three selected fields and averaged. The number of dead cells was calculated as a percentage of total number of counted cells.

2.3. Atomic force microscopy (AFM)

Aβ aggregation performed in the absence and in the presence of NP in molar ratio 1:1 or 1:5 were imaged with modular atomic force microscope. Five microliter samples of Aβ (74 μM) incubated for 6 days at 37 °C in the absence and in the presence of NP (74 μM or 370 μM) in 50 mM phosphate buffered saline was deposited on freshly cleaved mica and dried in nitrogen. Maximal scanning area was 20.5 μm × 20.5 μm for samples of height below 2 μm. The silicon cantilever with an integrated thermal actuator and piezoresistive Wheatstone bridge detector was used as a nanoforce sensor [29]. Cantilever resonance vibrations were achieved by periodically dissipating power in the thin film heater structure. Sine signal from the piezoresistive bridge was amplified and converted to its true RMS (root mean square) value using a selective electronics. The surface topography measurements were taken using shear-force mode, where the probe vibrated perpendicularly to the sample surface. Constant distance between microtip and the sample was maintained using an analog PID controller. Measurements of the surface topography were performed in electroaccustically isolated chamber at room temperature and ambient pressure with 50% humidity. At least four regions of the mica surface were examined to ensure that similar structures existed throughout the sample.

2.4. Thioflavin T (ThT) binding

The time-course of aggregation of Aβ was monitored with ThT (Sigma, USA), a fluorofore that shifts excitation and emission maxima upon binding to amyloid fibrils [30]. Twenty microliter of 74 μM Aβ solution was added to 980 μl of ThT (5 μM ThT in 50 mM glycine–NaOH buffer, pH 8.5), and mixed in a Vortex. The fluorescence was read within 1–2 min in a Perkin–Elmer LS-50 spectrofluorimeter (λ_{exc} 440 nm, λ_{em} 485 nm). The final concentration of Aβ in the assay was 1.48 μM.

2.5. Circular dichroism (CD)

The samples were measured in 0.1 cm path-length cells at room temperature with Jasco-600 spectropolarimeter equipped with a computerized data processor. MiliQ water was used as a solvent. Spectra were recorded at 1 nm intervals over the wavelength range 180–260 nm. Results are expressed in terms of molar elipticity (θ) in units of degree cm^2 dmol^{-1}. All the spectra were obtained by subtracting water base-line spectra and smoothed by using the algorithm provided by Jasco. The peptide solutions: Aβ (74 μM), Aβ + NP (74 μM + 370 μM), NP (370 μM), were analysed at various time intervals over 6 days of incubation at 37 °C. The samples for CD measurements were diluted 4–8 fold to secure measurements in the far UV range.

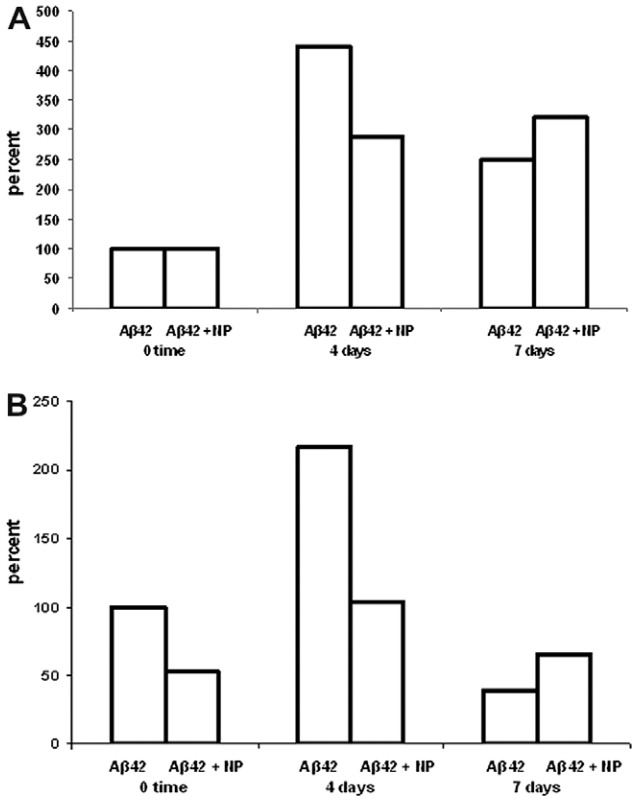

Fig. 1. In vitro fibrillogenesis of Aβ1-42 in the absence and in the presence of NP followed by the ThT fluorescence assay. Aβ (74 μM) was incubated for 4 days at 37 °C in the absence and in the presence of NP (370 μM). (A) Aβ1-42 with a low and (B) with a high 0 time ThT binding. Results shown are from a single representative experiment (two similar experiments were performed in triplicate). The results are presented as percentage vs ThT binding at 0 time assumed to be 100%.

3. Results

3.1. Thioflavin T binding

ThT is a dye that increases its fluorescence intensity upon binding the peptides with a beta-sheet conformation and is therefore used to estimate the relative amount of beta-sheets in a sample [30]. Therefore, to determine whether NP affects fibrillogenesis and amyloid formation, we measured fluorescence emission of ThT bound to amyloid fibrils.

In our studies we used commercially available batches of the amyloid peptide Aβ1-42. Different commercially available batches of Aβ1-42 can have different starting aggregation states and structure which will then in turn affect their solubility, aggregation rates, biological activities in solutions [31]. Nevertheless, all batches incubated at 37 °C for several days showed a significantly higher content of beta-sheeted fibrils than the amyloid at 0 time.

To determine whether NP affects Aβ fibrillogenesis, we measured aggregation of Aβ1-42 in the absence and in the presence of NP. We compared two batches of Aβ1-42, one with a low (Fig. 1A) and one with a high (Fig. 1B) fluoresecence at 0 time of incubation. In both cases, NP inhibited the aggregation of Aβ by 34–52%. Moreover, NP disrupted aggregates of Aβ present from the beginning (0 time) in solutions of some commercial preparations (Fig. 1B). During prolonged incubation (7 and more days) formation of insoluble amyloid-like structures of Aβ was observed. The presence of NP inhibited the precipitation (Fig. 1A and B). In control experiments, NP showed no binding of ThT.

3.2. Circular dichroism (CD)

Solutions of nonaggregated Aβ1-42 measured in water solutions display CD spectra characteristic for random coil [32]. Incubation of Aβ samples at 37 °C induces conformational changes of Aβ from random coil to beta-sheet structure, fibril formation and precipitation in an amyloid-like oligomeric beta-pleated sheet structure [32–36]. During incubation, the elipticity for CD band centered at 195 nm (max.) and 217 nm (min.) get stronger, corresponding to an increase in the amount of soluble beta-sheet structure [37].

CD spectra of our commercially obtained Aβ1-42 solubilized in water showed the presence of negative elipticity at 217 nm and positive one at 195 nm characteristic for β-sheet conformation. This indicated the presence of aggregates in our Aβ preparations. The changes observed during 1–6 days incubation at 37 °C indicated further transition of Aβ into beta-sheet conformation. CD spectra of water solutions of NP did not change during for 6 days (not shown). Comparison of the CD spectrum for Aβ + NP with the sum of CD spectra of Aβ and NP indicated that NP can directly interact with Aβ.

To study the effect of NP on the aggregation of Aβ1-42, transition into β-structure, we measured the CD spectra of water solutions of Aβ, Aβ + NP, and NP incubated at 37 °C for various time intervals.

As our commercial preparations of Aβ1-42 contained, ab initio, aggregates of Aβ, we decided to compare spectra of Aβ incubated in the absence of NP with the spectra of Aβ incubated in the presence of NP – difference spectra obtained by subtraction of buffer baseline spectra including NP. The effect of NP on the content of β-sheet

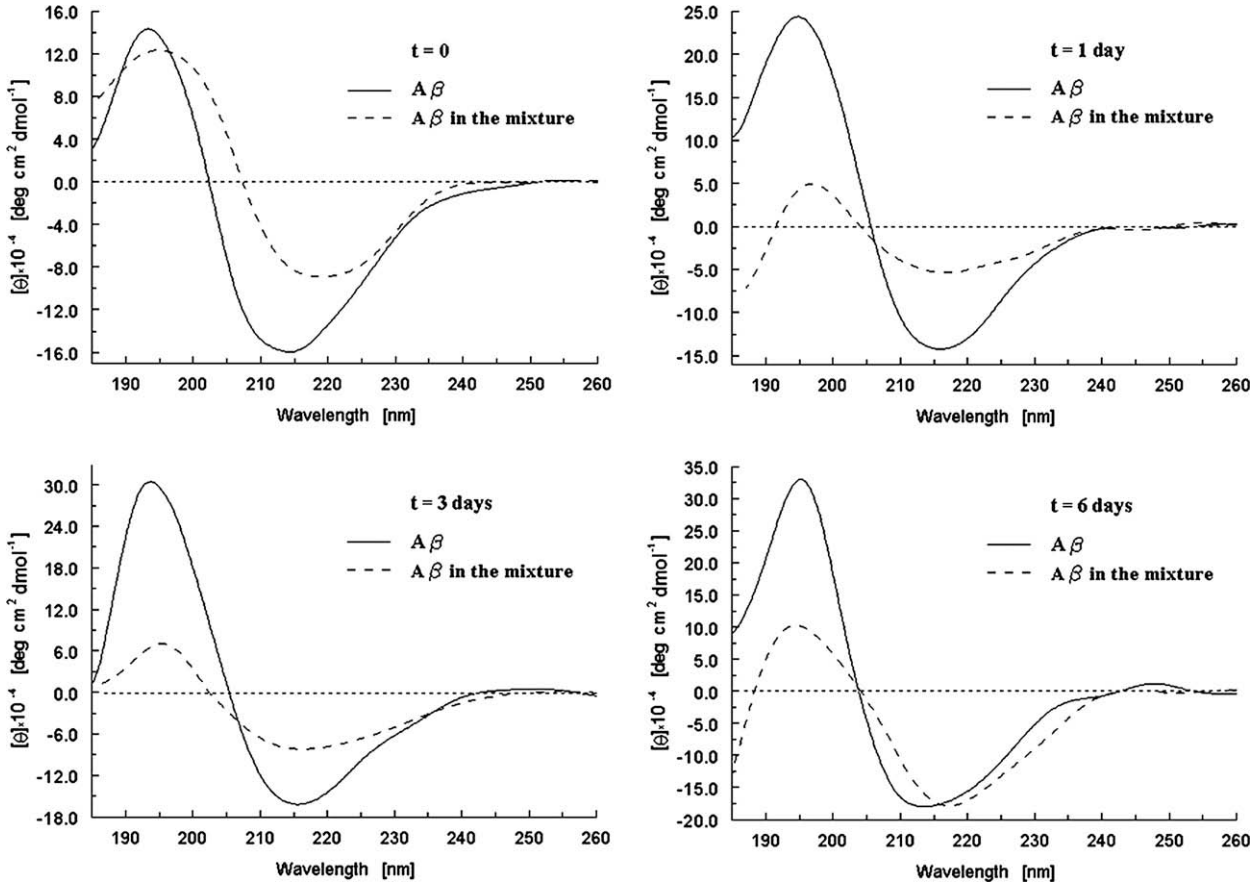

Fig. 2. Comparison of circular dichroism spectra of Aβ1-42 incubated in the absence and in the presence of NP (Aβ in the mixture). Spectral tracings for Aβ incubated for indicated times at 37 °C in the presence of NP were obtained after subtraction of base-line and NP elipticities from the Aβ + NP values (molar ratio Aβ:NP 1:5).

conformation of Aβ1-42 incubated at 37 °C is presented in Fig. 2. As is shown, NP caused an immediate reduction in the amount of β-sheet structure at time 0 and inhibited further transition of Aβ into β-sheet conformation during 6 days incubation. The results obtained showed that NP directly interacted with Aβ and may disassembly aggregates of Aβ and inhibit progressive aggregation and formation of β-sheet structure.

3.3. Atomic force microscopy (AFM)

Atomic force microscopy (AFM) provides quantitative and high resolution three dimensional morphological information which can hardly be obtained using other techniques. In our experiments we used 74 μM solutions of Aβ1-42 in 0.5 mM phosphate buffer, pH 7.0, incubated for 6 days at 37 °C in the absence and in the presence of NP (molar ratio NP:Aβ = 1:1 or 5:1).

In the case of commercial preparations of Aβ with a low content of aggregates and in the case of NP no presence of any specific structures was observed in AFM images at 0-time of incubation. However, 6 days incubation of Aβ induced formation of aggregates. Three-dimensional AFM image (Fig. 3) indicates the presence of coalescent aggregates of various extent of branching with the height of 34 nm. The aggregates exhibited a wide variety of shapes. In the two-dimensional images folded bands of aggregated Aβ which might represent the aggregated fibrils of Aβ and globular structures are visible. The amyloid nature of the aggregates was indicated by ThT binding and β-sheet-rich far ultraviolet CD. On the other hand, AFM images of Aβ incubated for 6 days in the presence of NP (molar ratio Aβ:NP = 1:1) showed only small spheroidal or ellipsoidal structures of height not exceeding 9 nm (Fig. 3). Solutions of NP showed after 6 days of incubation the presence of only a few small unstructured aggregates wit a height about 1 nm (data not shown).

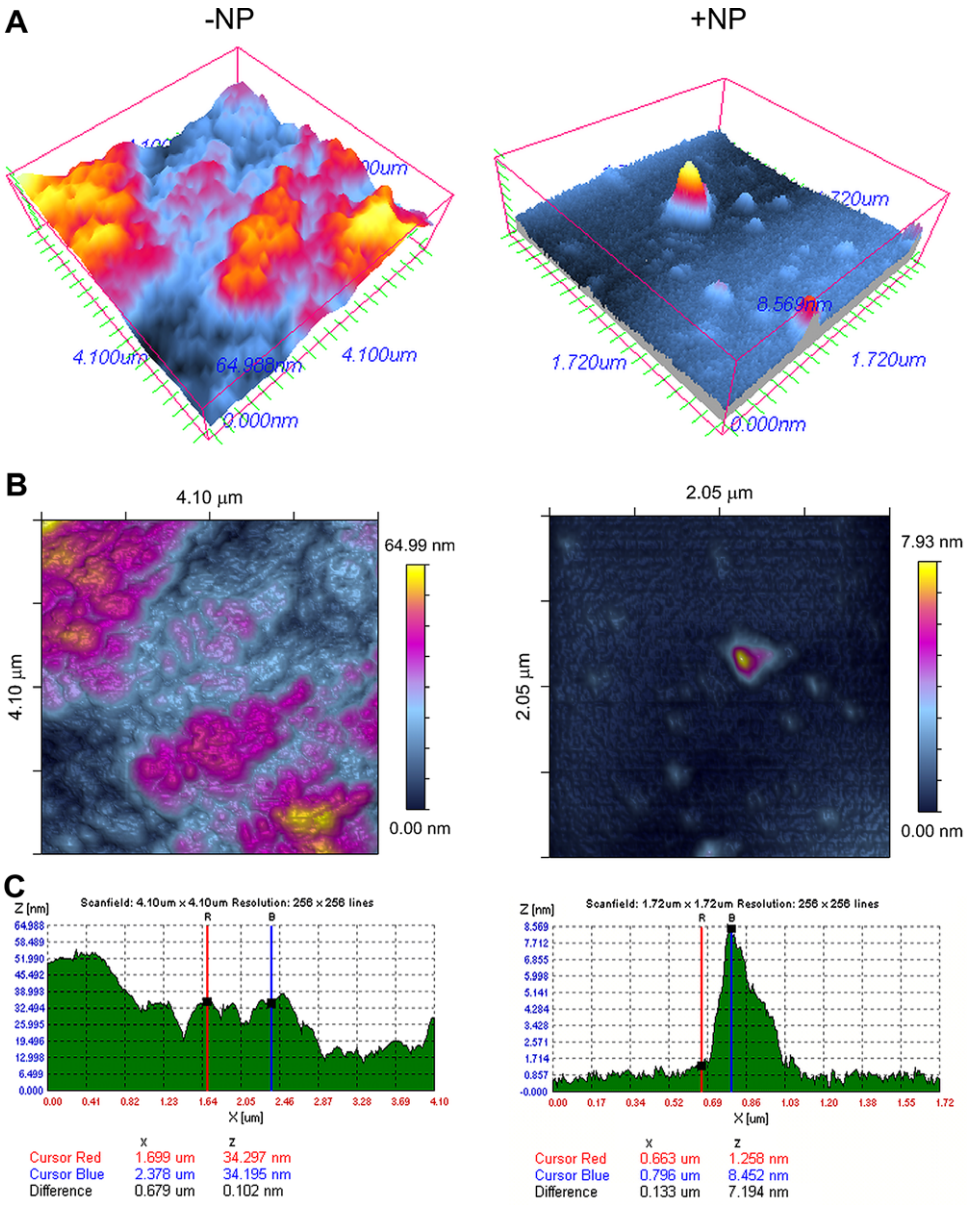

Fig. 3. AFM images of Aβ aggregates formed in the absence and in the presence of NP. Aβ1-42 (74 μM) was incubated for 6 days at 37 °C in the absence and in the presence of NP. Ex situ images of samples deposited on mica are presented as three dimensional image (A) two-dimensional image (B) and cross-section of AFM height image (C). Left side – in the absence of NP; right side – in the presence of NP.

Solutions of commercial Aβ1-42 preparations containing, ab initio, aggregated forms, showed at time 0 the presence of aggregates which further strongly aggregated within 6 days of incubation forming linear bands structures which further change into three-dimensional super aggregates with a height of 79 nm. However, when Aβ was incubated with NP (molar ratio Aβ:NP = 1:1) three-dimensional AFM image showed the presence of shortened linear, branched aggregates (not shown). The inhibitory effect of NP on Aβ aggregation was even more visible when the concentration of NP was enhanced (molar ratio Aβ:NP = 1:5). AFM image (Fig. 4) showed only the presence of small amorphous structures of height of 19 nm. Every sample surface was examined several times in different places using the scanning area of a few microns. Representative set of data, where we obtained average results, was shown in Figs. 3 and 4.

The results obtained showed that NP not only inhibited formation of large aggregates of Aβ but also induced deaggregation of Aβ aggregates already present.

3.4. Cell viability

Cell viability was measured by trypan blue exclusion assay. The results are presented as percentage of cells mortality in the presence of Aβ aggregated with or without of NP. As is shown in Fig. 5, in the presence of NP, both added to the cell simultaneously with βA or 24 hours before, toxic effect of Aβ is reduced up to about ¼ of the value obtained in the absence of NP. No toxic effect of NP was observed. The results obtained show that the reduction of Aβ peptides aggregation by NP is concomitant with the reduction of the cytotoxic effects of β-amyloid on PC-12 cells.

4. Discussion

One of a hallmark's of AD is the accumulation of depositions of insoluble amyloid fibrils as senile plaques in the brains of patients. Fibrillation proceeds via amyloid structures ranging from oligomers to protofibrils and fibrils. Transient, large globular or

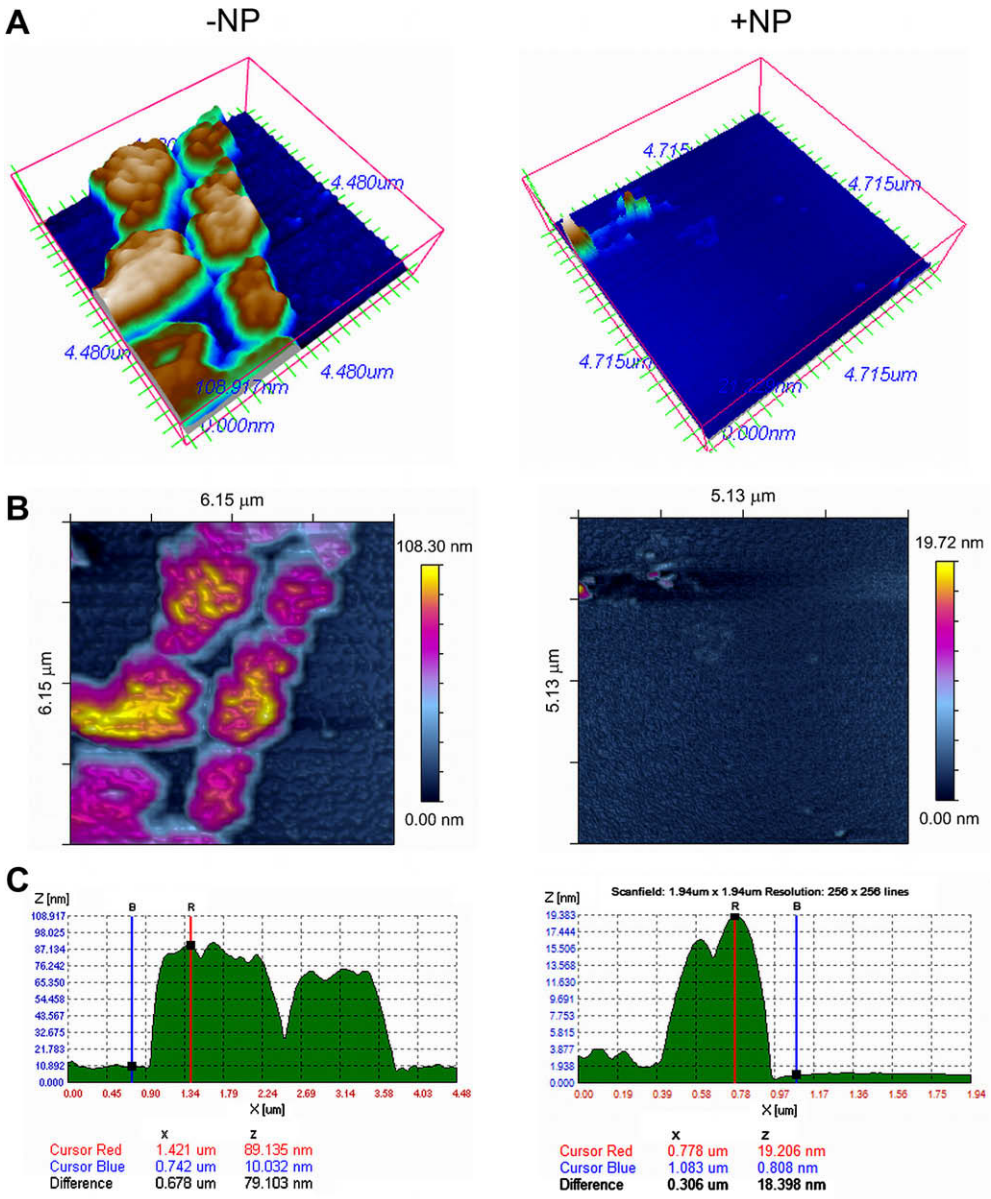

Fig. 4. Effect of the NP on deaggregation of the Aβ1-42 aggregates (AFM). Aβ1-42 (74 μM) preparation containing ab initio aggregated forms of Aβ was incubated for 6 days in the absence and in the presence of NP in a molar ratio 1:5. (A) Three-dimensional image, (B) two-dimensional image, and (C) cross-section of the height image. Left side – in the absence of NP; right side – in the presence of NP.

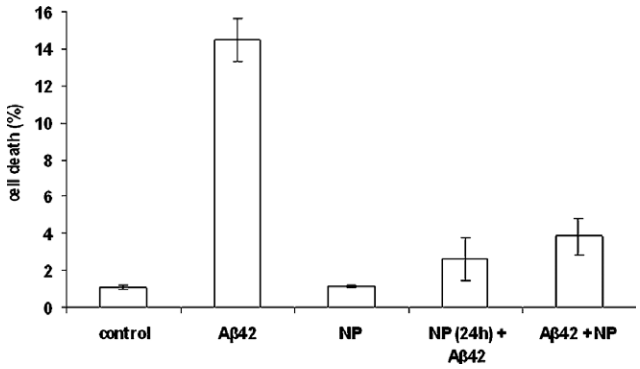

Fig. 5. Effect of NP of PC-12 cell viability. Cells were incubated for 72 h in the absence or in the presence of Aβ1-42 (1 μM), NP (1 μM), Aβ + NP (molar ratio 1:1) or pretreated with NP for 24 h and then treated with Aβ. The results presented are the means value of three independent experiments performed in triplicates. *Statistically significant differences versus amyloid β, $P \leqslant 0.05$.

amorphous aggregates can also be observed [31,32,47]. The plaques predominantly consist of aggregates of Aβ peptides [22]. Aggregated Aβ have been demonstrated to adopt a primarily β-sheet conformation in solution [22,33]. Soluble β-sheet structure is a precursor to the insoluble β-pleated sheet structure in amyloid plaques and is neurotoxic to neuronal cells [20,22,35]. As Aβ aggregates impair neuronal viability and function, drugs that inhibit the aggregation process may serve as effective therapeutics [38,39].

Tjernberg et al. [40] found that Aβ recognition element KLVFF (residues 16–20 of Aβ) interacts with full length Aβ and inhibits fibril formation. Subsequently, it was found that short synthetic peptides capable of binding Aβ, but not unable to become part of a β-sheet structure (β-sheet breaker peptides) may destabilize the amyloidogenic Aβ conformer and hence preclude amyloid formation [41–45].

It was of great interest to investigate whether the colostral proline-rich polypeptide complex which in form of sublinqually administered tablets, Colostrinin, shows positive effects in the case of AD, is able to affect the aggregation of Aβ. Recently, Schuster et al. [46] in experiments on aggregation of Aβ1-40 showed results suggesting that PRP/Colostrinin is a β-sheet breaker with an ability to prevent aggregation of Aβ1-40 and disrupt existing aggregates.

The aim of our studies was to investigate whether a NP, a component of PRP with known structure, immunotropic and procognitive properties [10,11,16], can affect the aggregation of Aβ1-42. Despite the small structural difference between Aβ1-40 and Aβ1-42, they display distinct clinical and biological behavior. Aβ1-42 displays enhanced neurotoxicity relative to Aβ1-40 and it forms aggregates and fibrils significantly faster than Aβ1-40. Amyloid peptide Aβ1-42 is the predominant component in parenchymal plaques [47,48].

Measurements of ThT binding, circular dichroism spectra, and atomic force microscopy indicated that NP was able to inhibit aggregation and β-sheet structure formation of Aβ1-42 in vitro. It also was shown that NP disassembled aggregates of Aβ already present in solutions of commercial Aβ1-42 preparations. Studies of CD spectra indicated that NP directly interacted with Aβ and acts as a β-sheet breaker. NP molecule possesses all properties expected from β-sheet breakers. It contains hydrophilic and hydrophobic segments and proline residues, as was described in the case of other β-sheet breakers [41,42,49]. The presence of Pro residues as β-sheet blocker residues is important for conformational flexibility and for destabilizing intermolecular β-sheets of Aβ aggregates [41,50].

There always is a problem how the results obtained from model system are related to process in vivo. In the case of PRP/Colostrinin, it is difficult to follow its pathways in the organism. The tablets of PRP/Colostrinin contain 100 μg of the PRP complex composed of more than 30 peptides. So the mechanism of action of PRP/Colostrinin can be deduced from the studies on model systems. PRP/Colostrinin was applicated to the AD patients in the form of sublinqual tablets. Oral-mucosal rout offers excellent accessibility, avoids degradation of protein and peptides that occurs as a result of oral administration, gastrointestinal absorption, and first-pass hepatic metabolism [51]. Peptides penetrating into the mucous membrane may gain access to the systemic circulation via a network of capillaries and arteries. It was shown, in various laboratories, that peptides can pass the blood–brain barrier using saturable transport systems (blood–brain, brain–blood), passive diffusion from blood to brain or saturable transport system after activation [52–54].

At present, we have no experimental evidence that PRP/Colostrinin can pass the blood–brain barrier. Neverthless, we can hypothesize that the PRP/Colostrinin might affect formation of the aggregates of the amyloid β peptides. Schuster et al. [46] showed that PRP/Colostrinin, even at low concentrations (2.5 nM and below) can affect the aggregation of Aβ and inhibit its aggregation.

It is assumed that aggregated forms of Aβ are responsible for the neurotoxic effects of this peptide [55]. Our results showed that one of components of the PRP/Colostrinin, the NP, can directly interact with Aβ and inhibit its aggregation. We can assume that PRP peptides, including NP, might cross the blood–brain barrier, enter the brain and interact with Aβ. Another possibility is that the PRP peptides might react with Aβ present in the serum affecting the exchange of Aβ between the blood and the brain and inhibiting the formation of Aβ aggregates. The second possibility was used as an explanation of the mechanism of action of anti-Aβ antibodies applicated to the AD patients [56].

The results presented in this paper suggest that PRP/Colostrtinin provides not only a combination of antioxidative and immunoregulatory effects which can all be important for the therapeutic effects of Colostrinin in AD patients, but also show that NP, the peptide component of PRP with the known primary structure and immunoregulatory and procognitive activities, can directly interact with Aβ and prevent formation of cytotoxic aggregates.

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